Mycorrhizae : physiology and genetics - Les mycorhizes : physiologie et génétique.
I^st ESM/I^er SEM, Dijon, 1-5 July 1985.- INRA, Paris, 1986

Induction of extracellular proteases in the ascomycete *Cenococcum geophilum*

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INTRODUCTION

Mycorrhizal fungi grow very often in soil layers where organic nitrogen compounds are present in large quantities. In such an environment it is possible that proteins constitute a significant source of nitrogen for fungi provided that these can produce the necessary depolymerase enzymes.

Several mycorrhizal fungi are able to utilize pure proteinsas a sole source of nitrogen (LUNDEBERG, 1970; ABU ZINADAH and READ, communication in this symposium). Moreover, protease activities in mycelial extracts and in culture filtrates of a few mycorrhizal organisms have already been reported (MELIN and HELLEBERG, 1925; LYR, 1963; RAMSTEDT and SÖDERHALL,1983). In addition, it appears that numerous proteases can be induced by the substrate as was demonstrated in *Neurospora crassa* (ABBOTT and MARZLUF, 1984).

Preliminary research in our laboratory has shown that the ectomycorrhizal fungus *Cenococcum geophilum* cultivated on Pachlewski's medium supplemented with ammonium as nitrogen source, was responsible for extremely low extracellular protease activity. A sensitive protease assay using fluorescein isothiocyanate was then developed and protease production was stimulated in circumstances where proteins were the only source of nitrogen available to the fungus.

MATERIALS AND METHODS

Growth conditions of Cenococcum geophilum The fungus (Strain Kiffer 1973) was collected from mycorrhizae. It was grown in shake culture at 25° C in Pachlewski's liquid medium containing nitrogen as diammonium tartrate (2.5 g/l) as described previously (MARTIN et al., 1983). Where indicated, the medium was modified by the deletion of ammonium salt. When included, proteins (Bovine serum albumin, gelatin, casein) were added as a filter-sterilized solution to a final concentration of 0.1%.

Measurement of protease activities: Enzyme activity was determined in culture filtrates either by spectrophotometry or by spectrofluorimetry. The first method was used with casein as substrate according to RAMSTEDT and SODERHALL (1983). Absorbance of the reaction mixture was measured at 280 nm and one unit of protease activity represents the amount of enzyme which released 1 µmol-tyrosine per min. at 37° C. The second method using fluorescence was carried out by using soluble fluorescein isothiocyanate-labeled bovine serum albumin (FITC-BSA). This substrate was prepared and

utilized according to the procedure described by TWINING (1984). Fluorescence was determined with a Farand spectrofluorimeter by using an excitation wavelength of 365 nm and an emission wavelength of 525 nm. The assays using the fluorescein label were at least 100 times, and even in some cases, 1000 times more sensitive than the measurement of cleaved casein peptides by absorbance at 280 nm.

Effects of some effectors: Calcium chloride was incorporated into the assay mixture at the appropriate levels. Inhibition of protease activity was studied by preincubating the enzyme for 30 min. at 20° C with diisopropyl fluorophosphate (Sigma).

RESULTS

Induction of protease excretion: During growth in a complete medium with nitrogen as ammonium, a very low level of protease activity was detected in culture filtrates (Fig. 1). Eight-day old mycelia were harvested by filtration and transferred to fresh nutrient solutions containing proteins as sole source of nitrogen. Controls were made by transferring the fungus to fresh media containing diammonium tartrate. Appearance of the enzyme activity began at 24 to 30 hours in thalli grown under inducing conditions, whereas those grown under control conditions secreted very little proteolytic activity into the growth medium (Fig. 1). The excretion of proteases continued nearly linearly for 10 days before beginning to level off. Gelatin was a more efficient inducer than BSA and casein.

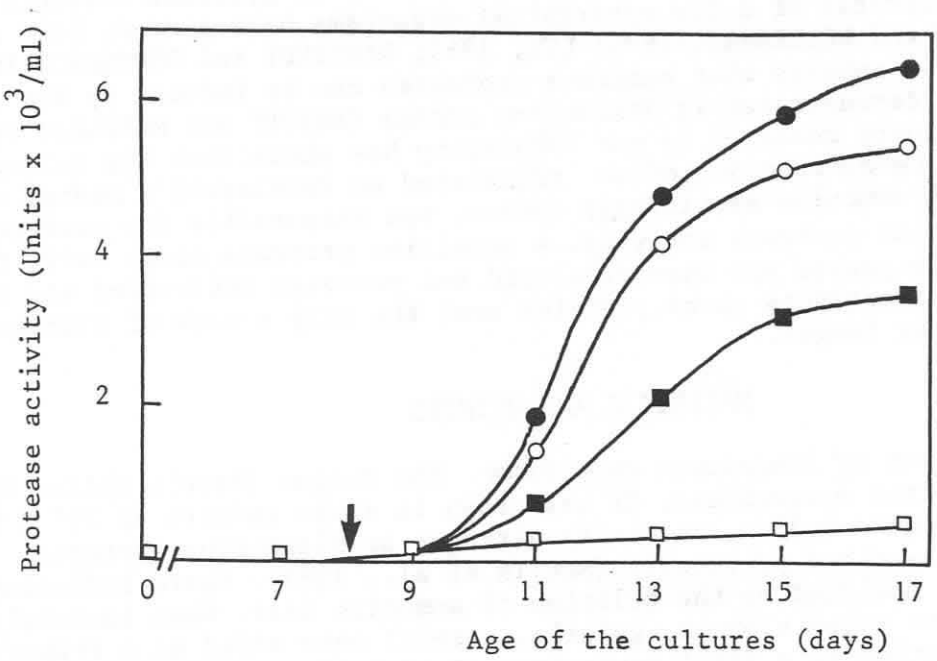

Figure 1: Changes in protease activity after transfer of the fungus to fresh media containing gelatin (●), BSA (○), casein (■) and ammonium (control) (□). Transfer is denoted by the arrow. Samples were harvested at 2-day intervals and the extracellular growth medium was assayed for protease activity by the casein assay at pH 11.

Similar inductions of proteases were obtained when the fungus was permanently grown in the same culture medium. In this case, the addition of proteins was performed after depletion of ammonium (data not shown).

Properties of the proteases:

Effect of pH: Maximum activities of the extracellular proteases were detected at pH 8.2 and 11 (Fig. 2). A low level of activity was sometimes observed around pH 4 but it always remained negligible.
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alkaline proteases.

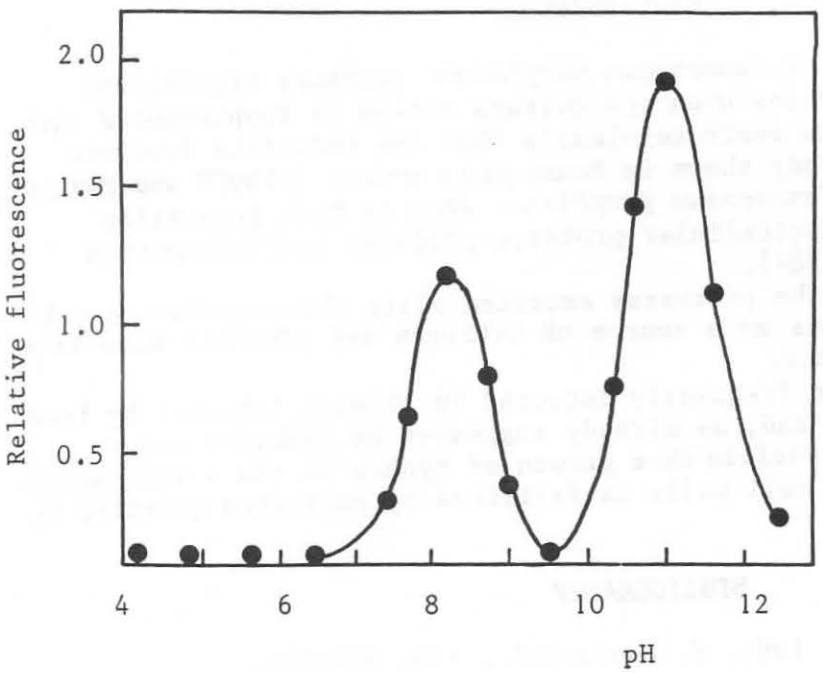

Figure 2: Effect of pH on the activity of the proteases. Enzyme activity was assayed by fluorescence. Samples were harvested after 8 days of induction by gelatin.

Effect of calcium: Both proteases were totally dependent on calcium for activity. The optimum concentrations of this cation were 1.5 mM and 3 mM for the enzymes operating at optimum pH of 8.2 and 11 respectively (Fig. 3).

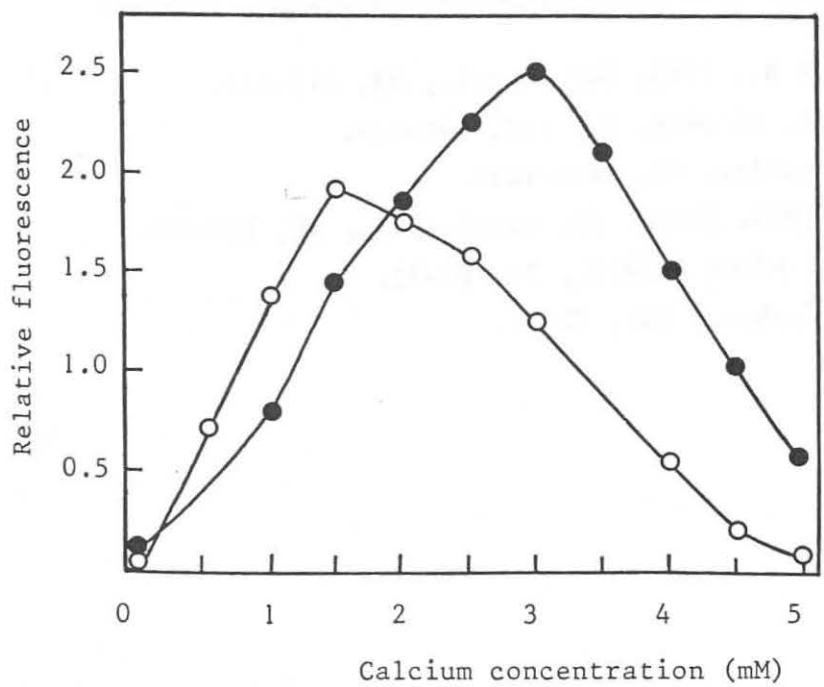

Figure 3: Effect of calcium on the activity of the proteases. Protease activity was assayed by fluorescence at pH 8.2 (○) and at pH 11 (●). Samples were harvested after 8 days of induction by gelatin.

Effect of protease inhibitor: The protease activities were strongly reduced by diisopropyl fluorophosphate 0.5 mM and completely inhibited at a concentration of 5 mM (results not shown). These data suggest that the induced extracellular proteases of *Cenococcum geophilum* belong to the serine protease group.

CONCLUSION

These results indicate that *Cenococcum geophilum* secretes significant amounts of alkaline proteases when the culture medium is supplemented with proteins. It remains to demonstrate clearly that the induction involves *de novo* synthesis as already shown in *Neurospora crassa* (ABBOTT and MARZLUF, 1984). The proteases of *Cenococcum geophilum* seem to have properties similar to that of the extracellular protease produced by *Cladosporium cucumerinum* (ROBERTSEN, 1984).

It is likely that the proteases excreted allow the organism to utilize extracellular proteins as a source of nitrogen and probably also as a source of carbon and sulphur.

Proteases have been frequently detected in tissues infected by fungi (KUC, 1962; PORTER, 1966) and, as already suggested by RAMSTEDT and SODERHALL (1983), it is plausible that growth of hyphae of the ectomycorrhizal organism between root cell walls is facilited by proteases excreted by the fungus.

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